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Biosynthesis of bisbenzylisoquinoline alkaloids in cultured roots of *Stephania cepharantha*

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Cultured roots of *Stephania cepharantha*, which are rich sources of bisbenzylisoquinoline alkaloids, were fed ^{14}C-labelled tyrosine, tyramine or dopamine. While tyrosine was well incorporated into the bisbenzylisoquinolines, tyramine and dopamine were poorly incorporated. Incorporated tyrosine was shown to be decarboxylated and stored as tyramine in the roots, then gradually converted to the bisbenzylisoquinolines. Tracer experiments using [3-^{13}C]tyrosine demonstrated that tyrosine was specifically incorporated into the corresponding sites of aromoline, which verified that aromoline was composed of four molecules of tyrosine. The ratio of ^{13}C-enrichments of C-4 and C-α in (*R*) and (*S*) halves of aromoline was the same within experimental limits. This indicated that the two coclaurine units must have one and the same biogenetic origin.

Bisbenzylisoquinoline biosynthesis; Aromoline; Root culture; *Stephania cepharantha*

1. INTRODUCTION

Bisbenzylisoquinoline alkaloids are structurally constructed of two monomeric benzylisoquinoline units joined by one or more ether linkages. Tracer experiments using whole plants or excised branches have shown that coclaurine and its *N*-methyl derivatives may act as bisbenzylisoquinoline precursors [1–4]. In a series of studies of protoberberine biosynthesis using cell-free extracts of cell cultures from the *Berberidaceae* and *Papaveraceae* families, it has been established that the benzylisoquinoline skeleton is formed from two molecules of tyrosine [5,6]. Therefore, bisbenzylisoquinoline alkaloids are thought to be formed from four molecules of tyrosine. Many problems remain to be elucidated in bisbenzylisoquinoline biosynthesis. For example, what is the direct substrate for dimerization and how is the *R*-half synthesized, because in protoberberine biosynthesis, the absolute configuration of synthesized benzylisoquinoline was established to be the (*S*)-form [5].

Plant cell cultures capable of producing the desired secondary products are excellent materials for biogenetic studies because cultures can be grown under defined conditions and the time required for product synthesis under cell culture conditions is much shorter than in field-grown plants. Cultured roots of *Stephania cepharantha* are an excellent source of bisbenzylisoquinoline alkaloids, containing more than 2% aromoline, more than 1% berbamine and smaller but still substantial amounts of isotetrandrine and homoaromoline [7,8]. All these dimers possess 1-(*R*), 1′-(*S*) absolute configurations [9].

In the present work we have fed *S. cepharantha* cultured roots with ^{14}C-labelled tyrosine, tyramine, dopamine and ^{13}C-labelled tyrosine and studied their incorporation into bisbenzylisoquinolines in an attempt to elucidate the fate of these early precursors in bisbenzylisoquinoline biogenesis.

2. MATERIALS AND METHODS

2.1. *Plant materials*

Cultured roots of *S. cepharantha* were established as described previously [8]. Cultures were maintained in flasks containing SB5 medium (modified B5 medium) supplemented with 3% (w/v) sucrose, 10 μM IBA and 1 μM GA in the dark at 26°C on a gyratory shaker at 80 rpm. Roots were subcultured at three week intervals.

2.2. *Chemicals*

L-(U-^{14}C]Tyrosine (17.98 GBq/mmol), [7-^{14}C]tyramine (2.07 GBq/mmol) and [7-^{14}C]dopamine (2.07 GBq/mmol) were purchased from Amersham International, UK. L-[3-^{13}C]Tyrosine (99.8 atom %) was purchased from Isotec Inc., USA.

2.3. *Feeding procedure*

^{14}C-Labelled L-tyrosine, tyramine or dopamine (10^7 dpm) was applied to vigorously growing 15-day-old cultured roots of *S. cepharantha*. The roots were allowed to metabolize for 10 days at 26°C in the dark. Thereafter, roots were harvested and alkaloids were extracted and analyzed.

In the pulse-chase experiment, ^{14}C-labelled tyrosine (1.9×10^7 dpm) was applied to 10-day-old cultured roots. After 10 days of culture, about half of the roots were harvested (58.1 mg dry wt.) and the re-

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maining roots were subcultured to a fresh medium containing unlabelled tyrosine. After 10 days of culture half of the roots were harvested (34.7 mg) and the remaining roots were grown for another 10 days and harvested (26.8 mg). All roots harvested were treated as described below.

2.4. *Extraction and analysis of alkaloids*

Harvested roots were freeze-dried and soaked overnight in MeOH. The suspension was centrifuged for 5 min at 3000 rpm and the supernatant decanted and saved. The pellet was re-extracted with MeOH and centrifuged. The supernatants were combined and evaporated to dryness at 40°C. The dry residue was dissolved in 2.0 ml of 3% citric acid and the acidic aqueous solution filtered through filter paper into a glass tube and made alkaline (pH 10) with aqueous ammonia. A 1.0-ml portion of this alkaline aqueous solution was put onto Extrelut column (Merck art. 11738). After 10 min, 3.5 ml of $CHCl_3$ was passed through the column twice. The $CHCl_3$ extracts were combined, then evaporated to dryness at 30°C. The dry residue was dissolved in MeOH and analyzed by HPLC.

Alkaloid content was measured with HPLC at room temperature. The stationary phase was Develosil ODS-3 (150 × 4.6 mm) and the solvent 70% MeOH containing 0.2% aqueous ammonia. The flow rate was 0.35 ml/min. A short pre-column (30 × 4.6 mm) was placed between the injector and the separation column. All the alkaloids were detected by UV absorption at 282 nm. The R_ts of tyramine, aromoline and berbamine were 8.3, 19.0 and 24.2 min, respectively.

Radioactivity of the fractions separated by HPLC was measured in a toluene-based scintillator.

2.5. *Identification of tyramine*

The alkaline fraction extracted from roots cultured in SB5 medium containing 200 ppm of tyrosine was separated by HPLC as described above. The peak at 8.3 min was collected. The mass spectrum of the peak eluting at 8.3 min was identical to an authentic sample of tyramine.

2.6. *Isolation of ^{13}C-enriched aromoline*

Roots were cultured for 25 days in SB5 medium containing 200 ppm of L-[3-^{13}C]tyrosine and then harvested and freeze-dried. A 6 g sample of the roots was treated as described above. The basic residue (0.28 g) yielded 85 mg of aromoline as slightly yellowish crystals.

2.7. *Determination of isotopic excess of ^{13}C-enriched aromoline*

^{13}C-Enrichment was calculated from peak-height analysis of ^{13}C NMR of aromoline. ^{13}C NMR spectra were measured at 50.32 MHz using a Bruker AC-200P spectrometer. The sample concentration was 37.5 mg/ml in $CDCl_3$ with TMS as the internal standard. The measuring parameters were as follows; pulse width 3.6 μs (45° flip angle), number of scans 12 000, pulse delay 2 s.

3. RESULTS

3.1. *Feeding experiments with ^{14}C-labelled precursors*

Previous studies on protoberberine biosynthesis have shown that tyrosine, tyramine and dopamine are incorporated into the benzylisoquinoline skeleton. Degradation of the labelled benzylisoquinoline showed that tyrosine was incorporated to an equal extent into both the benzyl portion and the isoquinoline portion. On the other hand, tyramine and dopamine were predominantly incorporated into the isoquinoline portion [10]. Similar results were shown in bisbenzylisoquinoline biosynthesis [11].

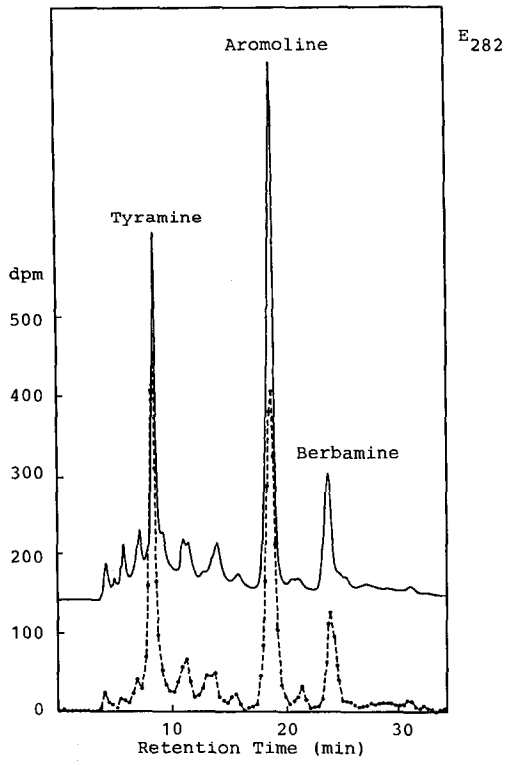

Fig. 1. HPLC separation of the basic fraction extracted from cultured roots of *S. cepharantha* administered L-[U-^{14}C]tyrosine. (•--•) Radioactivity of the fractions; (—) absorbance at 282 nm.

Roots were harvested 10 days after applying ^{14}C-labelled tyrosine, tyramine and dopamine to 15-day-old cultured roots of *S. cepharantha*. Incorporation of each substrate was 87–93% as determined by counting the remaining radioactivity in the medium. Crude alkaloids were isolated from methanol extracts of freeze-dried roots. In the methanol extracts, radioactivity derived from tyrosine and tyramine was sufficiently recovered but recovery from dopamine was very poor. In the crude alkaloids, radioactivity derived from tyrosine was recovered sufficiently for analysis by HPLC, whereas recovery of radioactivity derived from tyramine or dopamine was very poor (Table I).

Table I

Incorporation of potential precursors into cultured roots of *S. cepharantha*

Precursor	Incorporation (%)	Radioactivity recovered (× 10^3 dpm)	
		MeOH extract	Crude alkaloids
L-[U-^{14}C]Tyrosine	93.0	578	100
[7-^{14}C]Tyramine	91.3	654	6.6
[7-^{14}C]Dopamine	87.0	97	1.1

Table II

Changes in distribution of radioactivity in tyramine and bisbenzylisoquinoline alkaloids of *S. cepharantha* cultured roots after feeding with L-[U-^{14}C]tyrosine

Time[a] (days)	Amount (nmol/mg dry roots)			Specific activity (dpm/nmol)		
	Tyramine	Aromoline	Berbamine	Tyramine	Aromoline	Berbamine
20	12.8	18.1	6.4	83.1	67.6	62.5
30	12.7	19.0	7.3	31.8	32.9	30.1
40	17.9	22.1	8.7	8.4	17.6	15.1

[a]Amount of time roots were allowed to grow, including the period of preincubation, before harvesting

Table III

Effects of tyrosine on root growth and alkaloid formation in *S. cepharantha* root cultures.

Tyrosine conc. (ppm)	Growth index (*n*-fold)	Alkaloid content (% dry wt.)	
		Aromoline	Berbamine
0	22.4	2.83	1.29
40	17.2	2.83	1.29
200	18.4	2.49	1.10
400	14.3	2.37	1.07

Roots (0.1 g fresh wt.) were cultured for 29 days in 100 ml flasks containing 25 ml of SB5 medium with various concentrations of tyrosine. Each value is the mean of 3 replicates.

HPLC analysis of crude alkaloids extracted from cultured roots incubated with ^{14}C-labelled tyrosine showed that bisbenzylisoquinoline alkaloids were sufficiently labelled in proportion to the UV absorption (data not shown; Fig. 1 for reference). A time course study was undertaken to follow the change in distribution of radioactively labelled compounds. A solution of [U-^{14}C]tyrosine was fed to 10-day-old roots and roots were allowed to grow for periods varying from 10 to 30 days. The roots were then harvested and the crude alkaloids were extracted and subjected to HPLC analysis. A typical chromatogram is shown in Fig. 1. Three main peaks attributable to highly ^{14}C-labelled compounds are present. Two of the peaks are bisbenzylisoquinoline alkaloids (aromoline and berbamine), and the other peak was unknown. The unidentified peak was thought to be a key intermediate of bisbenzylisoquinoline biosynthesis. The unidentified com-

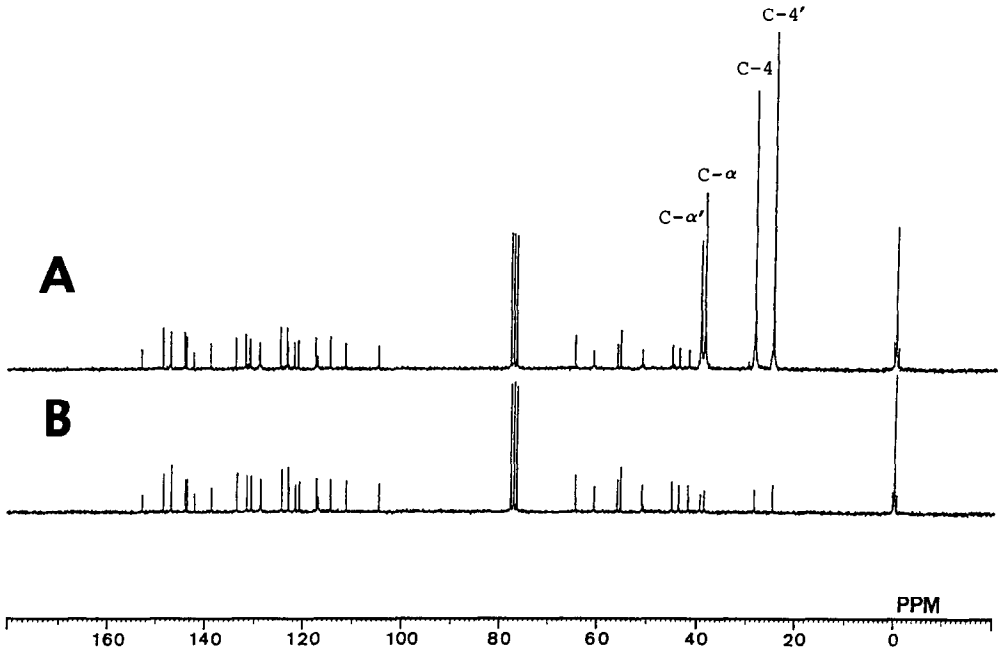

Fig. 2. ^{13}C NMR spectra of aromoline isolated from cultured roots of *S. cepharantha* administered L-[3-^{13}C]tyrosine. The reference sample (B) has normalized peak heights for comparison to the labelled aromoline (A).

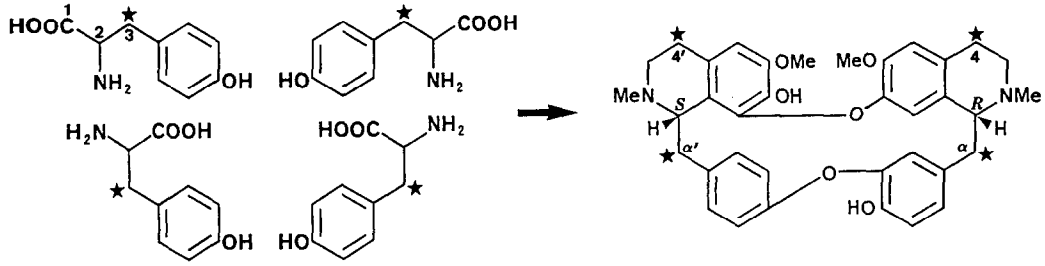

Fig. 3. Incorporation of four molecules of tyrosine into aromoline in cultured roots of *S. cepharantha*. * Position labelled.

pound was isolated and identified as tyramine from mass spectrum and chromatographic behavior (HPLC and TLC).

Table II compares the specific radioactivity of tyramine, aromoline and berbamine after 20, 30 and 40 days of culture. At the early stage, radioactivity was detected mainly in tyramine, then gradually transferred to the bisbenzylisoquinolines.

In these experiments, supplied tyrosine was incorporated into cultured roots and stored as tyramine, the decarboxylated product, which was gradually converted to bisbenzylisoquinoline alkaloids. To obtain a more precise assessment of the distribution of label derived from tyrosine and to elucidate how many molecules of tyrosine were incorporated into bisbenzylisoquinolines, feeding experiments of ^{13}C-labelled tyrosine were carried out as described below.

3.2. *Feeding experiments with ^{13}C-labelled tyrosine*

Preliminary experiments showed that less than 200 ppm of tyrosine did not significantly reduce cell growth or alkaloid formation (Table III). Roots were cultured for 25 days in SB5 medium [8] containing 200 ppm of [3-^{13}C]tyrosine and harvested. Six grams of dry roots was subjected to the isolation procedure and yielded 85 mg of aromoline as slightly yellowish crystals. ^{13}C NMR spectrum of ^{13}C-enriched aromoline is shown in Fig. 2A. Comparing the spectrum with that of unlabelled aromoline (Fig. 2B), only 4 signals were specifically enhanced. ^{13}C NMR spectrum of aromoline was previously assigned by Koike et al. [12]. According to their assignments, enhanced signals corresponded to the carbons at C-4 (δ 28.5), C-α (δ 38.3), C-4′ (δ 24.6), C-α′ (δ 39.4) of aromoline. These results are consistent with the prediction from biogenetic considerations [11] and verified that aromoline is composed of four molecules of tyrosine (Fig. 3). The ^{13}C-enrichments of the four signals were calculated from peak-height analysis of ^{13}C NMR of aromoline as follows; C-4 (16.24%), C-4′ (16.00%), C-α (10.49%) and C-α′ (9.31%). Carbons at C-4 and C-4′, as well as C-α and C-α′, were labelled equally. The ratio of ^{13}C-enrichment of the isoquinoline portion to the benzyl portion was calculated. The ratio was 1.55 and 1.72 when comparing to the (*R*)-half and the (*S*)-half, respectively.

4. DISCUSSION

The tracer experiments using ^{14}C-labelled compounds confirmed the incorporation of tyrosine into bisbenzylisoquinolines in cultured roots of *S. cepharantha*. The low incorporation of both amines, tyramine and dopamine, was contradictory to other reports using *Berberis* callus cultures [10]. This discrepancy may be due to the differences in the stage of differentiation between the plant materials. It might be more difficult to deliver the precursor to the alkaloid synthesizing cells in cultured roots than in calluses, especially when the precursor is chemically and biologically unstable as the free amine. Pulse-chase experiments using [U-^{14}C]tyrosine showed that tyrosine was incorporated into the cells and then stored in the form of tyramine, the decarboxylated product of tyrosine. HPLC analysis of the basic fraction extracted from cultured roots to which ^{14}C-labelled tyrosine was added did not show significant peaks corresponding to coclaurine (7.1 min) or *N*-methylcoclaurine (7.3 min). This implies that coclaurines are converted very rapidly to the bisbenzylisoquinolines or that the size of the metabolic pool of coclaurine and *N*-methylcoclaurine is small.

Tracer experiments using [3-^{13}C]tyrosine demonstrated beyond doubt that tyrosine was specifically incorporated into the expected sites of aromoline, without scrambling of the label to other carbons. These results verified the prediction from the biogenetic considerations of protoberberine biosynthesis and demonstrated that the benzylisoquinoline subunits of bisbenzylisoquinoline were synthesized in the same manner as protoberberines. Tracer molecules labelled with ^{14}C are used more frequently than ^{13}C-labelled tracers because of their increased sensitivity. Although ^{13}C-labelled tracers are less sensitive, they have the advantage that the location of the labelled position is easier to identify by ^{13}C NMR spectroscopy. In our cultures, it was possible to study the alkaloid formation with ^{13}C-labelled tracers because roots produced very large amounts of aromoline, which is isolated through a simple procedure [13]. Under our culture conditions, the precursor reached the alkaloid synthesizing cells without the necessity of being transported over long distances as in the case of whole plants.

Previous experiments have demonstrated that

tyrosine labelled both the isoquinoline and the benzyl portion with approximately equal efficiency. In our results, the ratio of ^{13}C-enrichment of isoquinoline portion to benzyl portion was about 6:4, which was similar to previous reports [10].

According to our results, the ratio of ^{13}C-enrichments of two positions, C-4 and C-α, in (*R*) and (*S*) halves of aromoline was the same within experimental limits. This showed that two coclaurine units must have one and the same biogenetic origin. According to Rueffer et al. [5], coclaurines were synthesized as (*S*)-forms by the stereospecific condensation of dopamine and phenylacetaldehydes in *Eschscholtzia* cell cultures, which are rich sources of isoquinolines. From their results and ours, (*R*) coclaurines might be derived from their corresponding (*S*) compounds, probably via dehydro-derivatives as was shown in morphine biosynthesis [14]. The fact that (*S*)-tetrahydroprotoberberine oxidase catalyzed the conversion of (*S*)-tetrahydrobenzylisoquinolines to the corresponding dehydroforms supports this hypothesis [15].

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